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Enhancement of B-cell translocation gene-1 expression by prostaglandin E_2 in macrophages and the relationship to proliferation

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SUMMARY

Although prostaglandin (PG) E_2 is known to suppress various macrophage functions, the molecular mechanisms by which that occurs are largely unknown. To understand better those mechanisms, differential screening of a cDNA library from PGE_2-treated macrophages was performed. Subsequently, the DNA sequence of a differentially expressed cDNA clone was determined and the cDNA was identified as B-cell translocation gene-1 (BTG1), a recently cloned antiproliferative gene. A two- to threefold increase in macrophage BTG1 expression was observed after PGE_2 treatment. PGE_1 and platelet-activating factor, but not leukotrienes B_4, and C_4, or lipopolysaccharide, also enhanced BTG1 expression. Furthermore, this effect was mimicked by dibutyryl cAMP which indicated the involvement of elevated cAMP in the PGE_2-mediated enhancement of BTG1. Moreover, there was an inverse correlation between BTG1 mRNA expression and macrophage proliferation; however, BTG1 alteration was not associated with macrophage tumoricidal activation. Thus, BTG1 may play a role in PGE_2-mediated inhibition of macrophage proliferation and not activation.

INTRODUCTION

Prostaglandin (PG) E_2 is thought to play a negative feedback role in regulating the extent and duration of a general immune response. PGE_2 not only prevents activation of natural killer (NK) cells, lymphokine-activated killer (LAK) cells and cytotoxic T lymphocytes, but also inhibits antibody production from B lymphocytes[1] and suppresses various macrophage functions such as tumour necrosis factor-α (TNF-α) production, I*a* antigen expression, and the proliferation of precursor cells.[2,3] PGE_2-mediated down-regulation of macrophage tumoricidal capacity is well documented.[4] However, the molecular mechanisms of PGE_2 actions on macrophages are largely unknown. One way to elucidate the mechanisms is to identify the gene(s) which may be regulated by PGE_2 and to characterize their gene products. From that, the molecular and cellular responses in which the gene products are involved may be studied. Differential screening techniques have been successfully used to study signal-induced genes in macrophages.[5–7] In our current study the same method was employed. The purpose of this study was to identify PGE_2-regulated genes and then to determine their potential role in PGE_2 alteration of macrophage functions. The sequence of a cDNA clone showing differential expression was determined and the cDNA was identified as BTG1, a member of a new family of antiproliferative genes.[8] As demonstrated with Northern blot analysis, PGE_2 selectively enhanced BTG1 expression possibly through the elevation of cAMP levels. Because of potential antiproliferative activity of BTG1 in transfected fibroblasts, we also examined the relationship between proliferation and activation of macrophages and BTG1 expression. The results presented here suggest that down-regulation of macrophage proliferation by PGE_2 may involve an increase in BTG1 mRNA expression.

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Abbreviations: BTG1, B-cell translocation gene-1; cAMP, cyclic AMP; CRE, cAMP-responsive element; LPS, lipopolysaccharide; LT, leukotriene; PAF, platelet-activating factor; PE, peritoneal exudate; PG, prostaglandin; PKA, cAMP-dependent protein kinase; TNF-α, tumour necrosis factor-α.

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MATERIALS AND METHODS

Reagents

PGE_2, PGE_1, leukotriene (LT) B_4, and LTC_4 were purchased from Cayman Chemical (Ann Arbor, MI). Lipopolysaccharide (LPS, *Escherichia coli*, serotype 0111:B4, phenol extract) and Bt_2cAMP were obtained from Sigma (St. Louis, MO), platelet-activating factor (PAF) was from Avanti (Alabaster, AL), maleic anhydride divinyl ether (MVE-2) was from Hercules (Wilmington, DE), tissue culture media from JRH Biosciences (Lenexa, KS) and serum from Hyclone (Logan, UT). Oligonucleotides were from the Protein Design Laboratory, University of California, Davis. Hamster anti-murine tumour necrosis factor-α (TNF-α) monoclonal IgG was from Genzyme

(Cambridge, MA), recombinant TNF-α (rTNF-α) and rabbit anti-murine TNF-α polyclonal for neutralizing were from Genzyme (Cambridge, MA); goat anti-rabbit, biotin-conjugated IgG and streptavidin-conjugated alkaline phosphatase were from Kirkegaard and Perry Laboratories (Gaithersburg, MD).

Macrophages

C57BL/6NCr female mice were obtained from Charles River Laboratories (Raleigh, NC). Animals were housed in autoclaved cages in a laminar flow hood and given sterilized water to minimize 'spontaneous' activation of macrophages. Cultures of responsive peritoneal exudate (PE) macrophages were prepared as previously described.[9] Briefly, 3 days before use, mice were injected intraperitoneally (i.p.) with 2·0 ml of sterile fluid thioglycollate broth. The resulting PE cells were harvested with Hank's balanced salt solution (HBSS) by peritoneal lavage, washed, and resuspended in Eagle's minimal essential medium, 5% heat-inactivated fetal bovine serum and 5 μg/ml gentamicin. Macrophages were counted and the concentration adjusted to an appropriate number then added to tissue culture plates. After adherence at 37° in 5% CO_2 for 90 min, the monolayers were washed with HBSS, treated with various agents for 0–24 hr, and total RNA was isolated. Resident macrophages were isolated from untreated mice and *in vivo* activated PE macrophages were isolated from mice injected i.p. with a combination of 500 μg MVE-2 and 10 ng LPS 5 days before use. Those doses of agents were previously shown to activate tumoricidal macrophages. For bone marrow-derived macrophages, bone marrow cells were obtained from mouse femurs, and cultured in RPMI-1640 supplemented with 10% L929 cell-conditioned medium and 2% fetal bovine serum for 7 days. After that time, cells were washed and total RNA was isolated. Greater than 98% of the final adherent population were macrophages as judged by morphology and phagocytosis of latex particles. RAW 264.7 cells [American Type Culture Collection (ATCC), Rockville, MD] were grown in Dulbecco's modified Eagle's medium with 5% calf serum. Steady-state levels of mRNA were determined by Northern blot analysis as previously described.[10] Unless indicated, thioglycollate-elicited responsive macrophages were used in the experiments.

Complementary DNA library construction

Thioglycollate-elicited responsive macrophages (3×10^7 cells/100 mm dish) were treated with PGE_2 (10^{-6} M) for 3 hr, and total RNA was isolated by acid guanidium thiocyanate–phenol–chloroform extraction.[11] Poly(A)$^+$ RNA was purified by selection with biotinylated oligo(dT) and streptavidin coupled to paramagnetic particles (Promega, Madison, WI). The cDNA library was constructed using a ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, first strand cDNA was synthesized from 5 μg of poly(A)$^+$ RNA with Moloney-murine leukaemia virus reverse transcriptase and *Xho*I-linked oligo(dT) primers in the presence of methyl nucleotides, followed by second-strand synthesis with RNAse H and DNA polymerase I. The double-stranded cDNA was blunt-ended with dNTPs and T4 DNA polymerase, the cDNA was ligated to *Eco*RI linkers, and then digested with *Xho*I. Sephacryl column-purified cDNA fragments were ligated into the *Eco*RI and *Xho*I sites of the bacteriophage λ vector arms (Uni-ZAP XR). Ligated vectors were packaged using Gigapack II Gold extracts, followed by amplification of the library in *E. coli* Sure strain to yield 5×10^9 plaque-forming units/ml.

Differential screening of the cDNA library

Synthesis of cDNA probes and screening of the cDNA library were conducted as previously described.[7] Briefly, poly(A)$^+$ RNA was isolated from either untreated macrophages or macrophages treated with PGE_2 as described above, and positive and negative complex cDNA probes were generated by first-strand cDNA synthesis reaction in the presence of (α-^{32}P)dGTP (800 Ci/mM, New England Nuclear, Boston, MA). Duplicate nitrocellulose lifts were made from each plate containing recombinant phages, and the filters were processed and hybridized with the probes as described.[12] Phage clones demonstrating differential hybridization between filters from positive and negative probes were rescreened in a similar way.

Northern blot analysis

Steady-state levels of mRNA were determined by Northern blot analysis as previously described.[9] Isolated total RNA (20 μg per sample) was electrophoresed in a 1·0% agarose gel containing 0·7% formaldehyde, and 20 mM 3-(*N*-morpholino)-propanesulphonic acid (MOPS), 5 mM sodium acetate, and 1 mM ethylene diamine tetra-acetic acid (EDTA). The RNA was transferred to Nytran (Schleicher & Schuell, Keene, NH). After ultraviolet cross-linking, the membranes were hybridized with the cDNA probe (10^6 c.p.m./ml). The membranes were then washed at 55° with 0·2 × SSC and 0·1% sodium dodecyl sulphate (SDS), dried, and exposed to X-ray film. In some experiments, autoradiograms were analysed with a scanning densitometer (CS-9000, Shimadzu, Concord, CA). For a cDNA probe, pBluescript phagemid harboring the cDNA insert was excised *in vivo* from a recombinant phage clone showing differential hybridization at the secondary screening. A 1·8 kilobase (kb) cDNA insert, liberated by a double digestion of pBluescript with *Eco*RI and *Apa*I, was random labelled with (α-^{32}P)dCTP (3000 Ci/mM, New England Nuclear) and an oligolabelling kit (Pharmacia, Piscataway, NJ). The β-actin probe was a 2 kb insert cloned into the PUC-9 plasmid at the *Pst*I site, and was similarly radiolabelled.

Determination and analysis of DNA sequences

Overlapping deletion clones were made by exonuclease III digestion of pBluescript containing the cDNA insert using the Erase-a-Base system (Promega). Dideoxy sequencing of each clone was performed with double-stranded DNA substrates and (α-thio-^{35}S)dATP (1500 Ci/mM, New England Nuclear) using the Sequenase version 2.0 DNA sequencing kit (US Biochemicals, Cleveland, OH). DNA sequence searches in GenBank and European Molecular Biology Laboratory data banks were performed with the FASTA program from Genetics Computer Group (University of Wisconsin Biotechnology Centre).

Serum starvation and stimulation of RAW 264.7 cells

RAW 264.7 cells were plated as indicated for measurement of proliferation. After 90 min, the media was changed to Dulbecco's modified Eagle's medium supplemented with 0·5% calf serum. In pilot studies, this amount of serum was determined to inhibit proliferation. The RAW 264.7 cells were visibly healthy and remained attached to the plates. After

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GGCACGAGCG GACGTGGAAA TGCAACTCTC GGGATCCTCG GAGGCTACCG
AGCTGGAGGC GGAGGCGGCT GGGGAGGTCC GAGCGATGTG ACCAGGCCGC    100
CATCGCTCGT CTCTCTCTGT CTCTCCTGCC GCCTCCTGGC TTGAAAATAA
CTTTTTTACT CTAAAGGAAA GAAAAAAAAT CCCCTAGAAC CAGTAGCCGT    200
CCGCGCCTCG GCCCTCGCTT CCTCTCGGCC GTCGGGTCTG GGCGAGAGGC
CCGGGTGGCC GCTTCGCCGT CGGGGCCGAG CCGGCCGCCC CGGGCCACCC    300
CCGGCCGCCG CCCCCATGCA TCCCTTCTAC ACCCGGGCCG CCACCATGAT
AGGCGAGATC GCCGCCGCGG TGTCCTTCAT CTCCAAGTTC CTCCGCACCA    400
AGGGGCTCAC GAGCGAGCGA CAGCTGCAGA CTTTCAGCCA GAGCCTGCAG
GAGCTGCTGG CAGAACATTA CAAACATCAC TGGTTCCCAG AAAAGCCGTG    500
CAAGGGATCA GGTTACCGTT GTATTCGCAT CAACCATAAG ATGGATCCTC
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AGGCTTCTCC CAAGTGAACT CACACTCTGG GTTGACCCCT ACGAAGTGTC
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TCTGGCACAA GATAGAGTGG TAATAATTTT TTTATTTCTT TCTTTTTTTT   1000
TTTTTTTAAT TTAGTGACAG TGCCATAGTT TGGACAGTAC CTTTCAGTGA
TTTAAATAGC CTGTGAGTCC AAGGAAAGGA TCACTTTATT TGCTAGGGAG   1100
TGAAGTCGAA GGGTGGTTTC AGTTTCTCCC AGACCTTATA CCCAATTTGT
CACAGCAGTC CCTTTAAGGA AATTCTGTCT TTCAAAGAAC CCTCTTTCGC   1200
AGTCAACCTT GCAGGGGAAT TTGCACTATT ACACTTGAAA GTTCTCAGTA
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TGAGTGGAGT AAACTTCTTA TTTTTAGAAA GTGGAGGTCT GGTTTGTAAC   1600
TTTCCTTGTA CTCAATTGGG TAAGAGTCTT TCCCACAAAC CACCATCTAT
TTTGTGAACT TTGTTAGTCA TCTTTTATTT GGTAAATTAT GAACTGGTGT   1700
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TATATTTTGG ATGAGAAATT TTTCTTCTCT CTATAATAAA TTGTTCGGCA   1800
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Figure 1. Nucleotide sequence of mouse BTG1 cDNA. Numbers on the right indicate nucleotide positions. The polypeptide coding region is underlined and the polyadenylation signal is double-underlined. The coding region is based on the assumption that translation begins at the first start codon in frame. The final sequence was determined from both strands. This nucleotide sequence reported in this paper is located in GenBank with accession number L16846.

addition of media with 10% serum at day 6, the cells were able to re-enter the cell cycle (data not shown). After 3 days of serum starvation, the media were changed to the same formulation with 10 ng/ml LPS added and cells were incubated for an additional 24 hr. Supernatants were collected and cells were harvested for Northern blot analysis.

Treatment of RAW 264.7 cells with BTG1 antisense

RAW 264.7 cells were plated and stained as described for the proliferation assay. After adherence of the cells, 14-mer phosphorothioate antisense or sense oligonucleotides directed to bases 316–329 were added, in triplicate, at a final concentration of 10 μM. Media and oligonucleotides were replaced on day 2.

Proliferation of macrophages

Proliferative capacities of the different macrophage populations were assessed by crystal violet staining. Adherent macrophages, prepared as described above, were incubated for up to 6 days. Bone marrow cells were first cultured in RPMI-1640 containing L929 cell-conditioned medium and fetal bovine serum for 7 days as described above. The adherent monolayers were then detached and transferred to new plates, and adhered for 90 min. RAW 264.7 cells were plated at 10^5 cells/well in 24-well plates with complete medium. After various periods of time, cell numbers were quantified by washing and staining the monolayers with 0·2% crystal violet in 2% ethanol. The optical density (OD) was determined on a microplate spectrophotometer at 570 nm.[13]

TNF-α enzyme-linked immunosorbent assay (ELISA)

The TNF-α ELISA was performed as previously described.[14] Briefly, microtitre plates were incubated overnight at 4° with hamster anti-murine TNF-α monoclonal IgG. After rinsing then blocking with 10% calf serum in HBSS, wells were again washed and TNF-α standard or samples in triplicate were added. After 1 hr wells were washed, incubated with a rabbit polyclonal antiserum against murine TNF-α then washed and incubated with a goat anti-rabbit, biotin-conjugated IgG. The plate was then washed, incubated with a streptavidin-conjugated alkaline phosphatase, washed, and developed using *p*-nitrophenylphosphate. The OD was measured with an ELISA reader at 407 nm.

Statistical analysis

Data were analysed with either a two-tailed Student's *t*-test or one-way analysis of variance (ANOVA). Pairwise comparisons

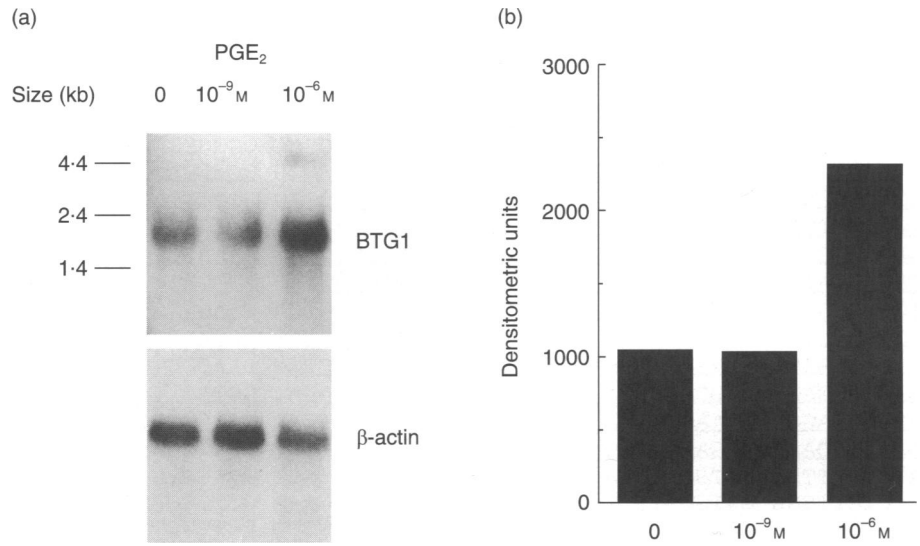

Figure 2. Enhancement of BTG1 expression by PGE_2. Thioglycollate-elicited responsive macrophages were treated with indicated concentrations of PGE_2 for 3 hr, and total RNA was isolated to evaluate the level of BTG1 mRNA by Northern blot. The autoradiograms (a) were analysed by scanning densitometry, and arbitrary densitometric units were determined (b). All densitometric data were normalized to β-actin.

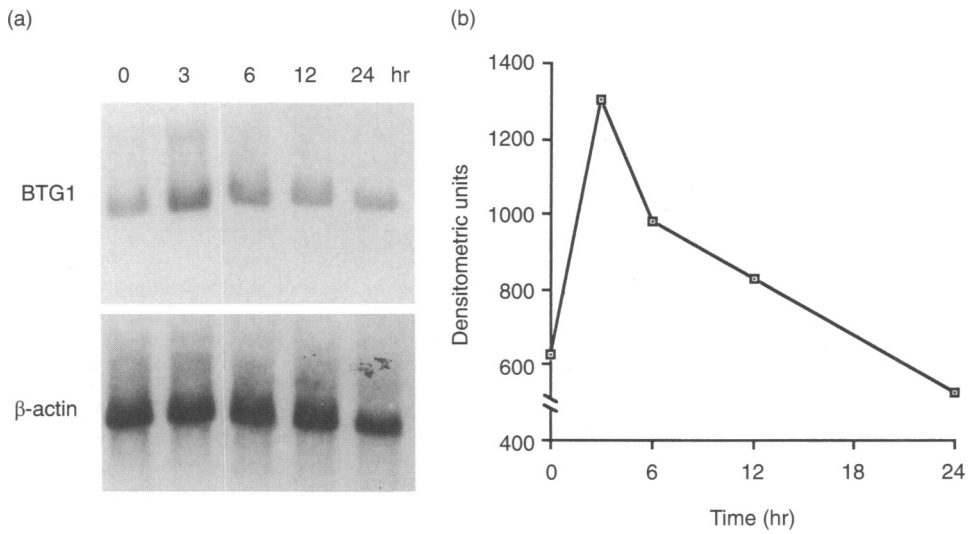

Figure 3. Kinetics of BTG1 mRNA accumulation in macrophages in response to PGE_2. RNA was isolated from macrophages at various times after treatment with 10^{-6} M PGE_2. RNA was subjected to Northern blot analysis. The autoradiograms (a) were scanned, and densitometric units determined (b).

were made using the Bonferroni inequality test. Differences were judged to be significant when $P<0{\cdot}05$.

RESULTS

Differential screening of the cDNA library from PGE_2-treated macrophages

A cDNA library was prepared in a bacteriophage vector with cDNA made from responsive macrophages that were treated with PGE_2 for 3 hr. That time-point was selected because PGE_2 induction or enhancement of gene expression probably occurs within 3 hr after exposure to the signal.[5] To isolate genes for which transcription was induced or enhanced by PGE_2, approximately 50 000 phage clones were screened by differential hybridization with cDNA probes generated from PGE_2-treated and untreated macrophage mRNA. After secondary screening, one clone showed differential hybridization. The cDNA insert from that clone was used for further studies.

Nucleotide sequence of the cDNA clone showing differential hybridization and its identification as BTG1

The DNA sequence of overlapping subclones made by exonuclease III digestion were determined by the dideoxy chain termination method (Fig. 1). Subsequently, the sequence of a 1·8 kb cDNA was compared to the sequences in GenBank and EMBL nucleotide sequence library data banks. There was a 100% identity in a 573 bp overlap with the previously reported

mouse BTG1 cDNA sequence (EMBL accession number Z16410). However, that mouse BTG1 cDNA sequence lacked most of the 5′ and 3′ untranslated regions when compared to the sequence of our clone. The putative human and mouse BTG1 proteins were found to be 100% identical and maintain 97% identity at the nucleotide level.[8] Similar to the human sequence, mouse BTG1 cDNA contained a long AT-rich 3′ untranslated region which may be important in post-transcriptional regulation of the gene.[15,16]

Enhancement and time–course of BTG1 expression by PGE_2

Differential expression of BTG1 in untreated and PGE_2-treated macrophages was confirmed by Northern blot analysis (Fig. 2). Macrophages constitutively expressed BTG1 transcripts of approximately 1·8 kb; 3 hr of treatment with 10^{-6} M PGE_2 increased BTG1 mRNA levels more than twofold. The mRNA level slowly decreased to the original level by 24 hr (Fig. 3). In contrast, treatment with 10^{-9} M PGE_2 showed no effect.

Effects of lipid metabolites, dibutyryl cAMP, and LPS on BTG1 expression

Besides PGE_2, other potent lipid mediators, including PGE_1, LTB_4, LTC_4, and PAF, may be produced by macrophages. These lipid mediators have also been shown to modulate macrophage functions and they are synthesized primarily by macrophages through closely related biochemical pathways.[17] Moreover, they often share a regulatory role in immune and inflammatory responses. Thus, we sought to determine whether those autocoids would alter BTG1 expression in macrophages as compared to PGE_2. Of those lipid metabolites, PGE_1 (10^{-6} M) and PAF (10^{-6} M, 10^{-5} M) significantly enhanced BTG1 expression, whereas LTB_4 or LTC_4 did not (Fig. 4a and c). Because an increase in intracellular cAMP levels has been demonstrated to be involved in the suppressive action of PGE_2 in macrophages, we next sought to determine whether the BTG1 mRNA level was altered by Bt_2cAMP. It was enhanced by 10^{-3} M or 10^{-4} M Bt_2cAMP (Fig. 4b), but not 10^{-6} M (Fig. 4c). We also tested the effect of LPS on BTG1 expression, because LPS has been shown to influence the expression of a number of genes in macrophages and LPS may induce PGE_2 production.[5] The BTG1 mRNA level was not significantly altered after 3 hr by the dose of LPS (100 ng/ml) (Fig. 4d), which induced new gene expression and activated macrophages for tumoricidal function.[9]

Correlation between expression of BTG1 and proliferative capacity of macrophages

Transfection experiments have previously indicated that human BTG1 negatively regulated proliferation of NIH3T3 cells.[8] Thus, we next assessed the relationship between the level of BTG1 expression and the proliferative capacity of macrophages. For that, three populations of macrophages in different activational states were used (Fig. 5). Because these macrophage populations may have different proliferative capacities, levels of BTG1 expression in each population were thought to be different. MVE-2-activated macrophages showed the highest level of BTG1 mRNA and the lowest proliferative capacity, whereas bone marrow macrophages had the lowest

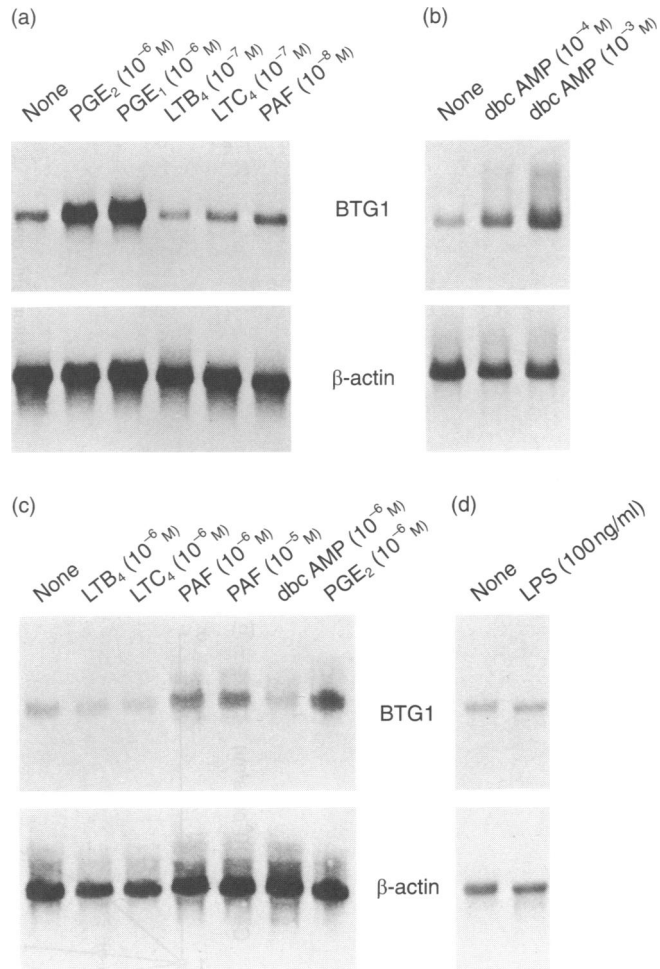

Figure 4. Effect on BTG1 expression of lipid mediators, dibutyryl cAMP and LPS. Macrophages were treated for 3 hr with either PGE_2 (10^{-6} M), PGE_1 (10^{-6} M), LTB_4 (10^{-7} M), LTC_4 (10^{-7} M), PAF (10^{-8} M) (a), dibutyryl cAMP (dbc AMP) (10^{-4} M, 10^{-3} M) (b), LTB4 (10^{-6} M), LTC4 (10^{-6} M), PAF (10^{-6} M), PAF (10^{-5} M), dibutyryl cAMP (10^{-6} M), PGE_2 (10^{-6} M) (c), or LPS (100 ng/ml) (d), and BTG1 mRNA levels determined by Northern blot. Data are from four experiments in which each experiment was repeated three times.

level of BTG1 and the highest proliferative capacity. After treatment of RAW 264.7 cells with a BTG1-specific antisense oligonucleotide, the level of proliferation was greater than in cells treated with a control, irrelevant oligonucleotide (Fig. 6). At day 3, proliferation of RAW 264.7 cells treated with the BTG1 specific antisense oligonucleotide was significantly ($P<0·05$) different than proliferation of cells treated with the nonsense oligonucleotide.

BTG1 expression was associated with proliferation and not activation of RAW 264.7 cells

Steady-state levels of BTG1 mRNA were not in RAW 264.7 cells treated for 3 hr with LPS, but was significantly enhanced twofold after 24 hr (Fig. 7). That treatment also inhibited proliferation (Fig. 7). Because RAW 264.7 cells were activated but stopped proliferating in response to LPS, we sought to determine the relationship of BTG1 expression to proliferation

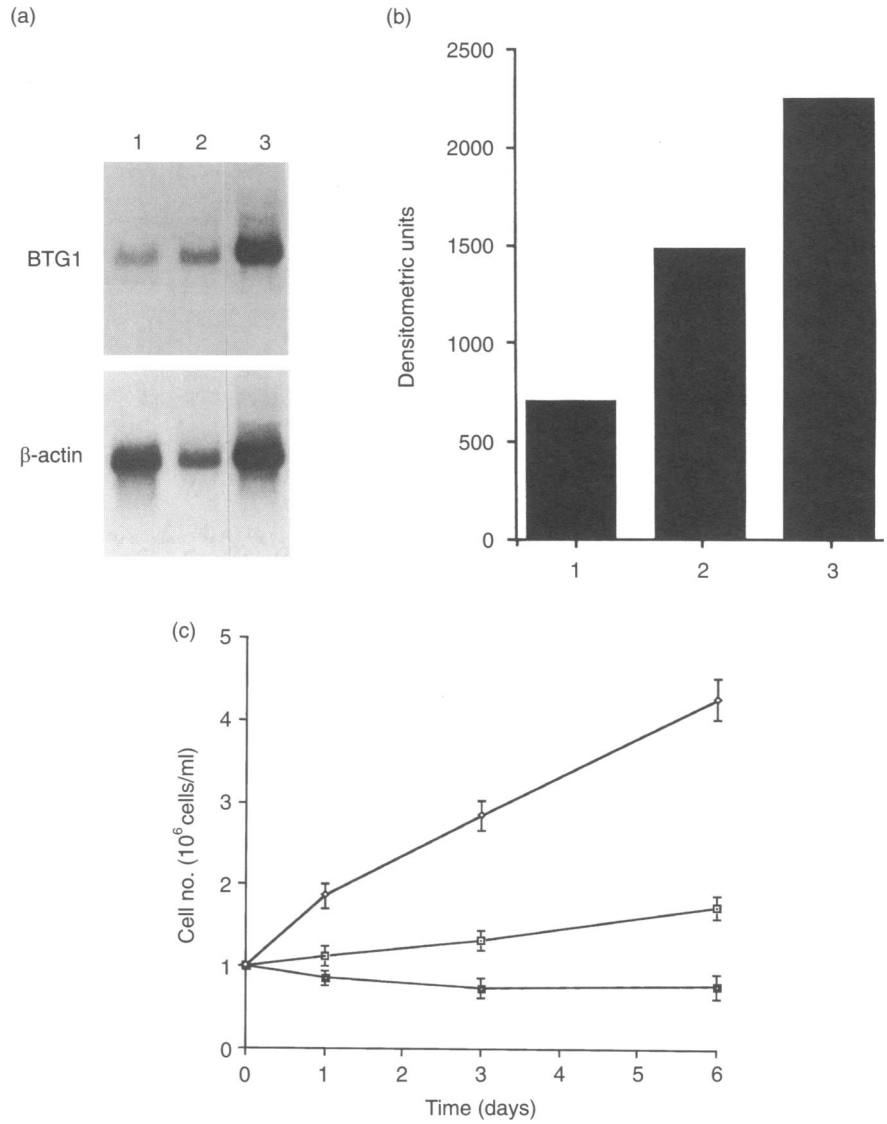

Figure 5. BTG1 expression and proliferation in different macrophage populations. (a) Bone marrow-derived (lane 1), resident (lane 2), and MVE-2-activated macrophages (lane 3) were prepared as described in the Materials and Methods. After 90 min adherence on culture plates, RNA was isolated from each population of macrophages. BTG1 mRNA levels were determined by Northern blot. (b) The autoradiograms were scanned, densitometric units determined and normalized to β-actin. (c) Proliferative capacity of each macrophage population was evaluated by crystal violet staining of cells. At 0, 1, 3 and 6 days after plating on culture dishes, macrophage monolayers were washed, stained and cell numbers were determined by optical density. Results are mean $\pm$ SEM for $n=3$; $\diamond$, bone marrow-derived macrophages; $\boxdot$, resident macrophages; and $\blacksquare$, MVE-2 activated macrophages.

and activation. RAW 264.7 cells were serum starved for 3 days which completely inhibited their proliferation (data not shown). After addition of 10 ng/ml LPS, those cells which constitutively produced less than 2 ng/ml TNF-α were activated, as demonstrated by production of 60, 90 and 80 ng/ml of TNF-α after 6, 12 and 24 hr. However, there was no significant enhancement of BTG1 mRNA when cultured for up to 24 hr (Fig. 8). Thus, BTG1 enhancement was not necessary for activation of RAW 264.7 cells, but was associated with the inhibition of proliferation when serum was present.

DISCUSSION

PGE_2 is known to suppress a number of macrophage functions. One known effect of PGE_2 on macrophages is the inhibition of precursor cell proliferation.[3] In our current study, PGE_2 increased BTG1 expression, and macrophages with little proliferative capacity expressed a higher level of BTG1 mRNA compared to other macrophage populations capable of proliferation. Likewise, cells treated with antisense demonstrated the inverse relationship of BTG1 expression and proliferation. Similarly, overexpression of exogenous BTG1 in NIH3T3 cells resulted in inhibition of cell growth.[8] Based on these observations, we believe that PGE_2 may inhibit macrophage proliferation by increasing BTG1 expression. Historically, it was thought that maintenance of resident tissue macrophages was dependent on the daily influx of peripheral blood monocytes for population renewal and that no, or a very low level of, cell division occurs in that population.[18] Now, however, there is increasing evidence for local division of tissue macro-

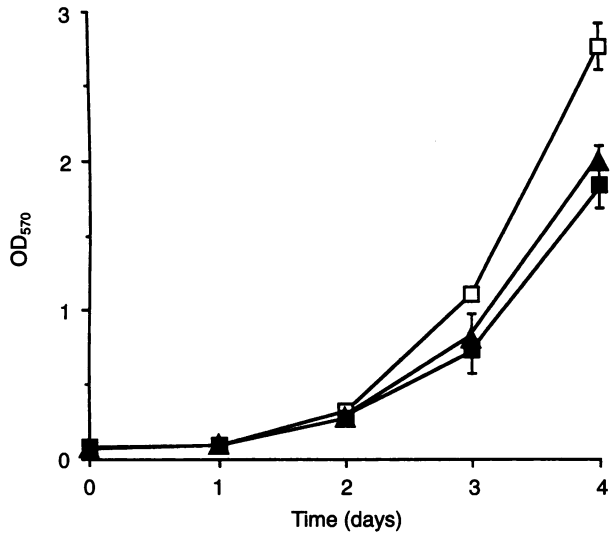

Figure 6. Treatment of RAW 264.7 cells with antisense. Cells were treated with antisense (□) or sense (▲) oligonucleotides or media (■). Crystal violet staining was performed each day. Values are means ± SD for $n=4$.

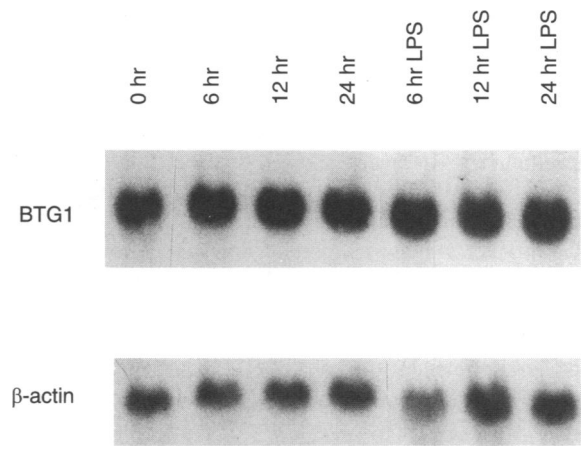

Figure 8. Northern blot of serum-starved RAW 264.7 cells after LPS treatment. Cells were treated the same as in Fig. 5, except they were harvested for Northern analysis at the indicated time.

phages.[19–23] PGE_2 may regulate this proliferative capacity of tissue macrophages in order to control immune and inflammatory responses in which prolonged or continuous proliferation of macrophages is required.

An increase in intracellular cAMP can serve an important regulatory role by either stimulating or suppressing the expression of selected genes in a variety of different cell types.[24,25] For example, cAMP-elevating agents strongly suppressed expression of TNF-α and the early-response gene, JE in macrophages,[24] while the same agents stimulated transcription of the tyrosine hydroxylase and somatostatin gene in PC12 cells.[25] Cyclic AMP is believed to mediate its regulatory effect on gene expression through a conserved cAMP response element (CRE). Elevation of cAMP activates cAMP-dependent protein kinase (PKA). Subsequently, this activated kinase post-translationally modifies the CRE-binding protein[25] or the CRE modulator[26] that binds to the CRE to either enhance or suppress gene transcription. PGE_1 and PGE_2 can attenuate the activational phenotypes of macrophages by receptor-mediated enhancement of adenylate cyclase activity which leads to elevation of intracellular cAMP levels.[27] As indicated in our current

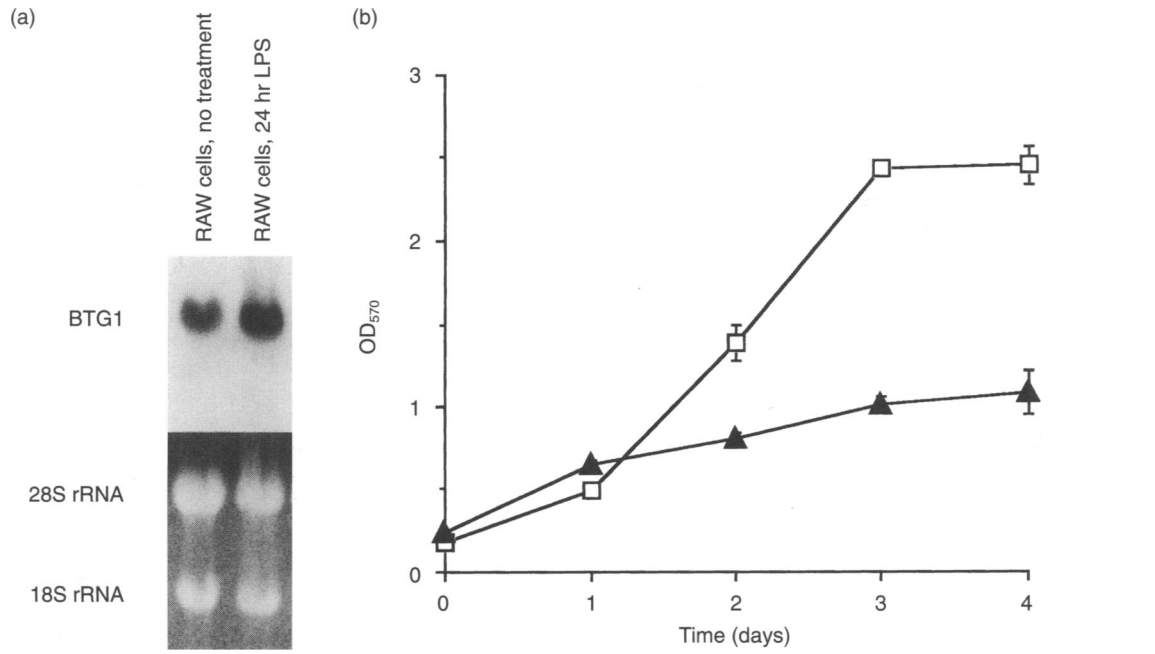

Figure 7. BTG1 expression and proliferation of RAW 264.7 cells treated with LPS. (a) Cells were stimulated with 10 ng/ml LPS for the indicated time and RNA was processed by Northern analysis. The blot was probed with BTG1. Ethidium bromide staining of the 28S and 18S rRNA was used to indicate equal loading of the RNA into the gel. The autoradiograms were scanned and densitometry units determined, and normalized to rRNA (data not shown). (b) Cells were grown in either media (□) or media plus 1·0 ng/ml LPS (▲). Crystal violet staining was performed each day. Error bars indicate SD.

study, both PGE_2 and PGE_1 as well as dibutyryl cAMP enhanced BTG1 expression. Furthermore, the human BTG1 gene has a CRE in the 5′ flanking region,[8] although the presence of the mouse equivalent has yet to be described. Thus, PGE_1 and PGE_2 may enhance BTG1 expression through elevation of intracellular cAMP levels followed by activation of PKA.

Among the different populations of macrophages, MVE-2-activated macrophages expressed the highest level of BTG1 mRNA, which was correlated with their lowest proliferative capacity. This indicates that one function of BTG1 in macrophages was related to antiproliferative activity. Considering the high proliferative capacity of bone marrow-derived macrophages, the level of BTG1 in these macrophages was expected to be lower than for resident macrophages. High levels of BTG1 mRNA expression in MVE-2-activated macrophages could be due to either *in vivo* exposure to their own secreted PGE_2 or PGE_2-independent signals involved in macrophage activation such as lymphokines. However, there may be additional BTG1-independent mechanisms by which proliferation of macrophages is controlled. Since three different populations of macrophages were used for the proliferation study, it is possible that BTG1 may be regulated in a different way in each population. Nevertheless, treatment of RAW 264.7 cells with BTG1-specific antisense supports a direct inhibitory role for BTG1 in proliferation. Although the steady-state level of BTG1 mRNA was not significantly affected by leukotrienes, other lipid mediators may influence BTG1 expression by similar or different mechanisms than PGE_2. For example, we demonstrated that PAF enhanced BTG1 expression, and PAF has been previously shown to increase intracellular cAMP as well as PGE_2 in human peritoneal macrophages.[28] This suggests that PGE_2 and PAF influenced BTG1 expression by similarly increasing intracellular cAMP. MVE-2-activated macrophages had a higher level of BTG1 mRNA as compared to other macrophage populations in different activational states. We, however, cannot draw firm conclusions from this correlative data about the relationship between BTG1 mRNA level and macrophage tumoricidal or other functional activities. This is because mechanisms for induction of a high level of BTG1 expression *in vivo* and the role of the BTG1 gene product in macrophage activation are unknown. However, a low level of expression in all macrophages is to be expected as it has been previously reported that the BTG1 message was expressed in almost all cells which have the capacity to proliferate.[8]

After LPS stimulation, macrophages become activated and stop proliferating. To study the relationship among BTG1, activation and proliferation a method was needed to determine whether the enhancement of BTG1 was associated with activation or inhibition of proliferation. This type of study has been done with other cell types by inhibiting proliferation through serum starvation.[29,30] This treatment prevented the endogenous antiproliferative genes from responding to antiproliferative signals. Genes involved in activation were still functional. The maximal amount of serum that would inhibit proliferation was used. The RAW 264.7 cells were serum starved, then stimulated with LPS for 24 hr. The concentration of LPS used was greater than the minimum required for inhibition of proliferation. Macrophages were activated, as indicated by TNF-α secretion. However, the level of BTG1 mRNA remained essentially unchanged. Therefore, BTG1 enhancement was not associated with activation but was associated with inhibition of proliferation. The mRNA for cyclooxygenase-2, the enzyme thought to be responsible for inducible PGE_2 secretion, has been shown to increase 24 hr after activation.[31] Furthermore, this PGE_2 secretion from macrophages has been associated with numerous suppressive phenomena, including resistance to subsequent stimulation and inhibition of proliferation.[20] Those phenomena caused by PGE_2 may be mediated in part by the BTG1 protein.

In inflammatory sites, heterogeneous populations of macrophages in different activational states may be present. Fully activated macrophages may secrete PGE_2, which in turn can act in an autocrine fashion or on other macrophage populations to inhibit proliferation. This may lead to suppression of immune or inflammatory responses which require recruitment and proliferation of blood monocytes at the site. Thus, enhancement of BTG1 expression and the ensuing inhibition of macrophage proliferation could be one way by which PGE_2 suppresses macrophage functions as well as local immune and inflammatory responses.

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